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Research Article

Micronuclei and chromosome aberrations derived from the action of Atrazine herbicide in *Allium cepa* meristematic cells

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ABSTRACT: Atrazine is an herbicide that causes serious damage to the environment and possibly affects the genetic material of organisms that come into contact with this agent, provoking mutations in their DNA. Although the herbicide Atrazine has been tested by various systems, a large number of deficiencies have been reported for many assays, and some evidence of its genotoxic effects still needs to be confirmed. The objective of the present study was to evaluate the herbicide Atrazine effects, analyzing the mitotic index alterations and the induction of chromosome aberrations, using the *Allium cepa* test system. Fifty *Allium cepa* seeds were used for germination in Atrazine solutions at the concentration used in the field (0.125 ppm) and at progressively lower concentrations (0.062, 0.031 and 0.015 ppm). After 20 h, some roots were then collected from each assay, while the remaining roots were transferred to plates containing Milli-Q water and allowed to recover for 48 h. The herbicide reduced the germination indexes of seeds, the mitotic indexes of cells and induced alterations in the genetic material of the test organism, what suggests that this herbicide presents genotoxic effect at all concentrations tested.

KEY WORDS: Atrazine herbicide, cytotoxicity, genotoxicity, chromosome aberrations, micronuclei, *Allium cepa*.

1.INTRODUCTION

A wide variety of chemical substances with mutagenic potential, both natural and synthetic, have been investigated. Many of these substances are found in food, pharmaceutical drugs, agricultural pesticides, and complexes of domestic and industrial effluents. Many of these compounds are known to cause damaging hereditary changes in the genetic material that might not be immediately expressed (Vogel, 1982). Concern regarding the proliferation of chemical agents introduced in the environment that cause possible genetic alterations in different organisms has been one of the main reasons for the development of methods for the assessment of the mutagenicity of chemical substances (Brusick, 1987).

Biological toxicity, genotoxicity and mutagenicity assays are essential for the assessment of the response of live organisms to environmental pollution, as well as for the identification of the potential synergistic effects of various pollutants (Chauhan et al., 1999).

Most assays testing genotoxic and mutagenic substances are based on the evaluation of the induction of chromosome damage such as structural alterations, micronuclei formation, sister chromatid exchanges, and on the analysis of mutant genes or DNA damage. For this purpose, different organisms such as bacteria, plants and animals have been used in both in vitro and in vivo assays (Vogel, 1982; Leme and Marin-Morales, 2009).

Nowadays it is possible to estimate the genotoxic, mutagenic, carcinogenic and teratogenic effect of various agricultural pesticides employing relatively simple methods. Among these chemicals, Atrazine is one triazinic herbicide, which has been classified as moderately toxic, used before and after plant emergence. This herbicide is used for the control of weeds in asparagus, corn, sorghum, sugar cane and pineapple cultures (Popa et al., 1986). Atrazine is classified as a restricted use pesticide and it is currently evaluated the human and ecological health risks associated with its release into the environment (Bolle et al., 2004). Triazinic herbicides are among the pesticides most widely used in agriculture (Eldridge et al., 1999;).

Atrazine has been tested by various systems, but deficiencies have been reported for some of these assays, and some evidence of its in vivo cytotoxic and genotoxic effects still needs to be confirmed (Ribas et al., 1998; El-Ghamery et al., 2000; Kligerman et al., 2000; Fenton et al., 2002; Bolle et al., 2004; Ventura et al., 2008).

Cytogenetic assays are adequate to identify the harmful effects of substances at different concentrations and for different times of exposure. These assays, which are generally performed using test organisms, are commonly applied to the biomonitoring of the extent of pollution and to the assessment of the combined effects of toxic and mutagenic substances on organisms in the natural environment (Matsumoto et al., 2006; Leme and Marin-Morales, 2009).

Among higher plants, *Allium cepa* has been indicated as an efficient genotoxicity test organism. This efficacy is based on the characteristics of the proliferation kinetics of the plant, the rapid growth of its roots, large number of dividing cells, high tolerance to different culture conditions, availability throughout the year, easy handling, and the small number (2n=16) and large size of its chromosomes (Fiskesjö, 1985; Leme and Marin-Morales, 2009).

Allium cepa was originally introduced as a test organism in 1920 (Fiskesjö, 1985; Rank and Nielsen, 1993), and has since then been used to assess and classify the toxicity of chemicals present in the environment (Fiskesjö, 1985). This species has been used as a test plant indicator of mutagenicity by different investigators (Brusick, 1987; Rank and Nielsen, 1993; Ma et al., 1995; Khors et al., 1997; Rank and Nielsen, 1997; Smaka-Kincl et al., 1997; Kovalchuck et al., 1998; Rank and Nielsen, 1998; Matsumoto et al., 2006; Ventura-Camargo et al., 2011; Mazzeo and Marin-Morales, 2015).

Positive results obtained with the *Allium cepa* test should be regarded as an indication that the tested chemical may also cause biological damage to other organisms (Fiskesjö, 1985). *Allium cepa* has been considered one of the best plant test systems established, which is routinely employed for the assessment of the genotoxic and mutagenic potential of chemicals in the environment due to its good correlation with mammalian test systems (Brusick, 1987). The sensitivity of the Allium mutagenicity assay is higher than 82% compared to the results obtained with rodents (Rank and Nielsen, 1993).

The aim of the present study was to evaluate the genotoxicity of the herbicide Atrazine, by the chromosome aberrations induction, using the *Allium cepa* test system.

MATERIALS AND METHODS

Tested substance

Atrazine (CAS n°. 1912-24-9; pure 97.7%) is a selective herbicide of the triazines chemical group and its composition is 2-cloro-4-ethylamine-6-isopropylamine-s-triazine. The doses used in the field range from 5 to 7 liters per hectare diluted in 200 to 400 liters of water depending on the culture.

Test-system, treatment solutions, and bioassays.

The biological material used as test system consisted of *Allium cepa* seeds (2n=16 chromosomes), which were submitted to germination at different Atrazine concentrations always at room temperature and on plates protected from light.

Fifty *Allium cepa* seeds were used for germination in Atrazine solutions at the concentration used in the field (0.125 ppm) and at progressively lower concentrations (0.062, 0.031 and 0.015 ppm). All assays were performed in duplicate in order to obtain a large sample size.

All seeds were submitted to germination in Milli-Q water until their roots reached a length of 2 cm and then transferred to plates containing the different Atrazine concentrations, except for the control (in that the seeds were submitted to germination in Milli-Q water only), being always one plate for each concentration. All seeds were incubated for 20 h. Some roots were then collected from each assay, while the remaining roots were transferred to plates containing Milli-Q water and allowed to recover for 48 h. After this period, new roots were collected. All roots collected were fixed in Carnoy's solution diluted 3:1 (3 parts ethanol and 1 part acetic acid) for 6 to 18 h.

For cytological analysis, meristems were submitted to acid hydrolysis in 1 N HCl at 60°C for 11 min, followed by washing in distilled water, and stained with Schiff's reagent for 2 h in the dark. All slides were obtained by gently squashing the meristems between the slide and coverslip. The coverslips were removed with liquid nitrogen and the slides were mounted in Enthelan for subsequent analysis.

Dividing cells showing the following abnormalities were observed: prophases with loss of genetic material, metaphase chromosome adhesions, C-metaphases, irregular anaphases (multipolar anaphases, laggarded anaphases and anaphase bridges), cells containing chromosome breaks, losses and adhesions, cells with telophase laggard, and cells with micronuclei. The Kruskal-Wallis test was used for statistical analysis.

RESULTS

The seeds germination indexes for the 20h treatment with the Atrazine herbicide in 0.015, 0.031, 0.062 and 0.125 ppm concentrations were 83, 78, 73 and 70%, respectively, lower than the milli-Q water (control) seeds germination index (95%). When the seeds and roots were transferred on plates containing only milli-Q water, for a 48h recovery period, seeds germination indexes previously treated with the 0.062 and 0.125 ppm Atrazine concentrations had remained practically the same ones (75 and 71%, respectively). However, seeds previously treated with 0.015 and 0.031 ppm (90 and 89%, respectively) showed germination indexes seemed to the control test.

The mitotic indexes of the cells derived of roots submitted to treatment with only Milli-Q water (control) were 25.3 and 24.1 for 20 h and 48 h, respectively. Differences were observed in the mean mitotic indexes of cells resulting from the exposure to the various Atrazine concentrations. Mitotic indexes were 16.92, 14.34, 16.69, and 15.03% for roots treated for 20 h with 0.015, 0.031, 0.062 and 0.125 ppm Atrazine, respectively. Mitotic indexes were 24.87, 23.5, 13.78 and 12.97%, respectively, when the roots were allowed to recover on plates containing only Milli-Q water for a period of 48 h (Table 1).

Table 1: Mitotic Indexes (MI) observed in *A. cepa* meristematic cells incubated for 20h in the presence of different Atrazine concentrations and after recovery period of 48h.

Treatment periods	Negative Control	Atrazine concentrations (ppm)			
		0.015	0.031	0.062	0.125
20 h	25.30%	16.92%*	14.34%*	16.69%*	15.03%*
Recovery-48h	24.10%	24.87%	23.50%	13.78%*	12.97%*

The frequency of aberrant cells in roots submitted to treatment with Milli-Q water (control) was 0.100 and 0.040 for roots treated for 20 h and 48 h, respectively. Differences were observed in the mean frequencies of cells with aberrations resulting from the exposure to the various Atrazine concentrations. The frequency of aberrant cells was 1.07, 1.28, 2.36 and 1.59% for roots treated for 20 h with 0.015, 0.031, 0.062 and 0.125 ppm Atrazine, respectively (Table 2). The frequency of aberrant cells for these same roots decreased to 0.53, 0.53, 0.30 and 0.80%, respectively, when the roots were allowed to recover on plates containing Milli-Q water for a period of 48 h (Table 2).

Table 2: Frequency of chromosome aberrations and micronuclei observed in *Allium cepa* cells incubated for 20 h in the presence of different concentrations of the Atrazine herbicide and after a recovery period of 48 h.

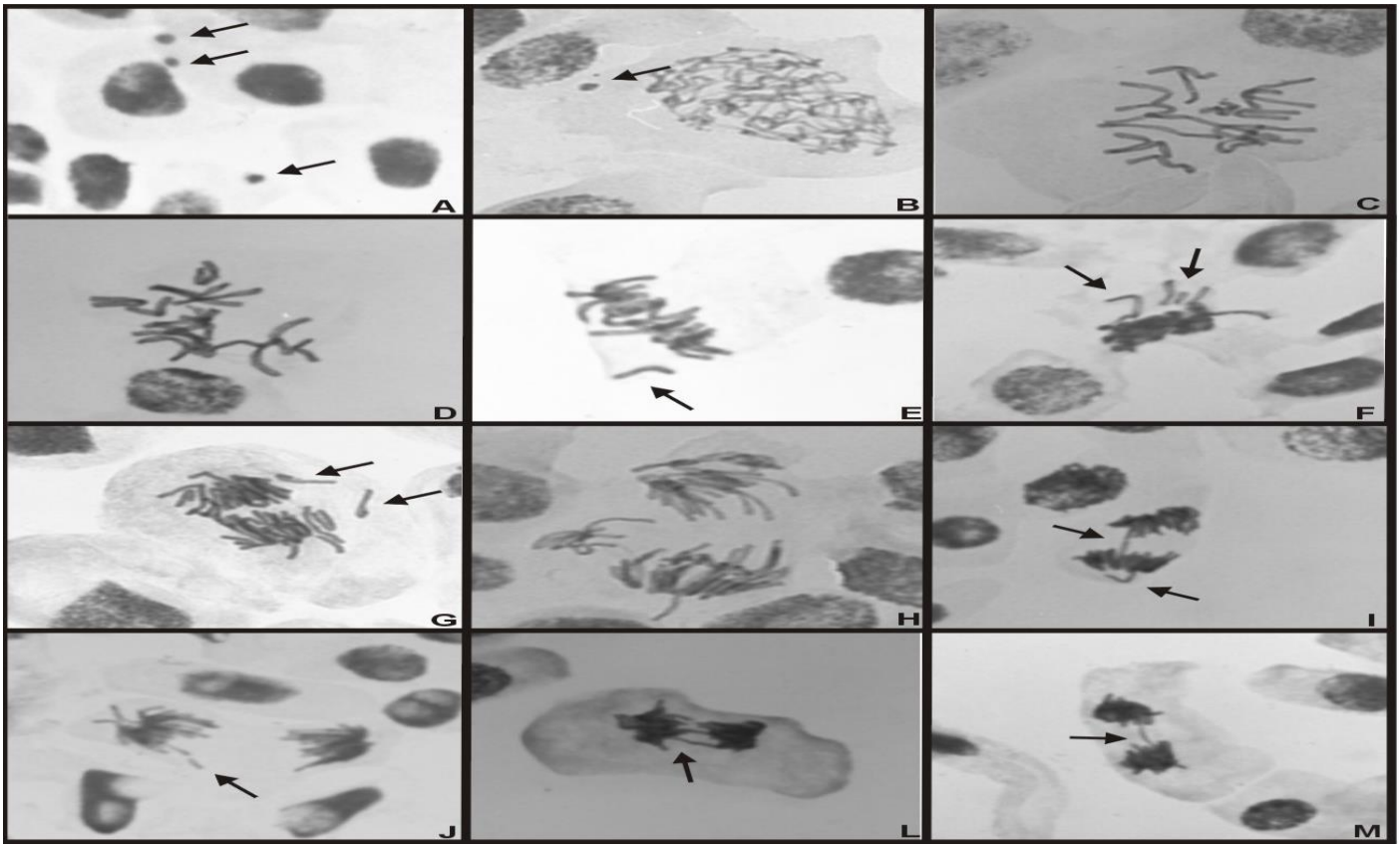
Cell Alterations	Negative Control		Atrazine concentrations (ppm)							
			0.015		0.031		0.062		0.125	
	20 h	48 h	20 h	48 h	20 h	48 h	20 h	48 h	20 h	48 h
Multipolar anaphase	0	0	0.065	0.030	0.050	0.095	0.005	0.045	0.215	0.017
Anaphase and/or telophase bridges	0	0	0.030	0.065	0.335	0.035	0.040	0.030	0.220	0.005
Chromosome break	0	0	0.015	0	0.100	0	0.010	0.005	0.055	0
Anaphase laggard	0.010	0	0.050	0.035	0.135	0.210	0.110	0.030	0.265	0.017
C-metaphase	0	0	0.240	0.075	0.225	0.080	0.090	0.030	0.120	0.405
Loss of genetic material uring prophase	0.010	0.010	0.214	0.030	0.115	0	0.145	0	0.075	0.025

Telophase laggard	0	0.010	0.090	0.035	0.055	0	1.670	0.060	0.290	0.055
Metaphase adherence	0	0	0	0.030	0	0.015	0.080	0.025	0.010	0
Chromosome loss	0	0	0.030	0	0	0.055	0.010	0	0.045	0
Micronuclei	0.080	0.020	0.340	0.225	0.250	0.045	0.205	0.080	0.300	0.275
Total of chromosome aberrations	0.020	0.020	0.730*	0.305*	1.030*	0.485*	2.155*	0.220*	1.290*	0.525*
Total of cell alterations	0.100	0.040	1.070*	0.530*	1.280*	0.530*	2.360*	0.300*	1.590*	0.800*
Total number of cells analyzed	11,415	11,571	9,975	10,345	10,579	9,518	10,249	10,740	11,028	10,105

The most frequent types of aberrations observed in cells submitted to the various Atrazine concentration for a period of 20 h and in cells recovered in Milli-Q water for 48 h were: multipolar anaphases, anaphase and/or telophase bridges, chromosome loss during metaphase and/or anaphase, anaphase laggard, micronuclei, C-metaphase, loss of genetic material during prophase, telophase laggard, and chromosome loss during metaphase and/or anaphase (Figure 1).

FIGURE 1: ***A. cepa* cells showing chromosome aberrations and micronuclei after treatment with different concentrations of the Atrazine herbicide.**

A. Interphase cells with micronuclei; **B**. prophase cell with a micronucleus; **C** and **D**. C-metaphase cell; **E**. metaphase cell showing chromosome loss; **F**. metaphase cell presenting adherence and chromosome loss and breaks; **G**. anaphase cell showing chromosomes losses; **H**. cell presenting multipolar anaphase; **I**. cell showing an anaphase laggard; **J**. anaphase cell with chromosome break; **L**. anaphase cell presenting chromosome bridges; **M**. cell in initial telophase showing a chromosome bridge.



The most frequent types of alterations observed in cells submitted to the herbicide Atrazine are related to the loss of genetic material (chromosome bridges, losses, breaks and laggards) (Table 2). Those losses are responsible by the presence of micronuclei in the interfasic cells of later generations.

The total frequency of chromosome aberrations observed for roots treated for 20h with 0.062 ppm Atrazine was significant level when compared to control.

DISCUSSION AND CONCLUSION

We observed that the higher the tested Atrazine concentration, higher was the inhibition of the seeds germination. Such results demonstrate the high toxic potential of the herbicide, especially to the 0.062 and 0.125 ppm Atrazine concentrations, that showed significant values when compared to the control test. Our results corroborate the researches performed by Marshall and Nel (1980), who verified a reduction in the seeds germination indexes of sorghum. Besides, El-Ghamery et al. (2000) also verified that 3.75 g/l Atrazine concentration reduced the mitotic division in *A. cepa* and *Vicia faba* compared to the respective negative controls.

After the recovery period, we observed an increase seeds germination indexes for the 0.015 and 0.031 ppm, but almost the same indexes of seeds germination for the 0.062 and 0.125 ppm Atrazine concentrations. Thus, a 48h period of recovery in milli-Q water seems to be efficient in minimizing the toxic effects of such herbicide in the two lower tested concentrations. On the other hand, for the 0.062 and 0.125 ppm concentrations Atrazine, the toxic effects were higher, showing that the recovery period performed was not sufficient to repair the toxic damages. Such toxic effects in the two tested higher concentrations could be irreversible to the organism test.

There was a significantly decrease of mitotic indexes of the roots submitted to the Atrazine herbicide concentrations for a period of 20 h when those were compared to the control test, demonstrating that the tested chemical can cause cytotoxic effects in *Allium cepa* and, therefore, for all exposed organisms to such herbicide. Moreover, we observed that higher the concentration tested, higher is the harmful in the cells treated. According to Ribas et al. (1998), the Atrazine herbicide induced cytotoxic effects in human, reducing significantly the cell proliferation of lymphocytes. After the recovery period with milli-Q water, for a period of 48h, we observed a good response of cells related to the cytotoxicity. Thus, we can infer that the recovery with milli-Q water was efficient to minimize the inhibition of mitotic indexes.

The total frequency of aberrant cells observed for all Atrazine concentrations tested and for the two treatments (20 h and recovery) was significantly higher than that obtained in the control test. Such data corroborate other studies that showed that Atrazine herbicide presents genotoxic potential for several organisms (Tennant et al., 2002; Bolle et al., 2004; Ventura et al., 2008; Marin-Morales et al., 2013).

The highest percentage of aberrant cells was observed for a concentration of 0.062 ppm, confirming the elevated genotoxicity of higher concentrations of this herbicide. The 0.062 ppm concentration induced the largest number of chromosome aberrations, and can thus be regarded as the diagnostic concentration of Atrazine genotoxicity among the concentrations tested.

Lower frequencies of aberrant cells were observed in all assays after the recovery period. Thus, a 48 h period of recovery in milli-Q water seems to be efficient in minimizing the induction of the chromosome aberrations of the herbicide, since a decrease in the frequency of aberrations, or even the disappearance of some aberrations, was observed after recovery.

Our results demonstrated the presence of various chromosome aberrations in the different assays. For example, multipolar anaphases were identified which are the result of malfunctioning of the mitotic spindle causing irregular chromosome distribution to more than two poles of the cell, in contrast to what is observed during normal cell division (Rank and Nielsen, 1997).

Other aberrations found were chromosome breaks during metaphase and/or anaphase, chromosome laggards, and anaphase and telophase bridges. Chromosome breaks can result in chromosome fragments and anaphase bridges can, the latter, deriving from translocations or simply from cohesive chromosome ends (Fiskejö, 1993). Bridges possibly also result from chromosome adhesions and, in this case, can be multiple and can persist until telophase. When chromosome bridges are secondary to structural rearrangements, they may result in chromosome fragments (Fiskesjö, 1993).

We also observed the presence of micronuclei, which might have been the result of a chromosome laggard during anaphase characterized by spindle malfunctioning or even of the presence of acentric fragments due to a clastogenic response (Sudhakar et al., 1998; Ventura-Camargo et al., 2011). Thus, chemical agents can induce micronuclei through spindle

disturbance or chromosome breaks. Bolle et al. (2004) analyzed the genotoxic effects of Atrazine herbicide on *Allium cepa* bulbs and indicated that chromosome breaks were the predominant lesions induced, which demonstrated the clastogenicity of Atrazine at concentrations that are likely to be encountered in water, a common site of Atrazine contamination.

We also propose that micronuclei might have been the result of both a chromosome laggard during anaphase and acentric fragments (Sudhakar et al., 1998; Matsumoto et al., 2006; Ventura et al., 2008; Leme and Marin-Morales, 2009). From our cytological observations that evidence the presence of anaphase chromosome bridges, we also suggest that micronuclei originated of acentric fragments should correspond to the lost telomeric regions involved on the break.

The presence of C-metaphases is another indicator of the genotoxicity of Atrazine. In the case of C-metaphases or C-mitosis, the nuclear chromosome is completely inactivated and thus no equatorial plate becomes organized and, consequently, centromere division is laggarded or even impaired. As a result, the presence of C-metaphase cells leads to a duplication of the number of chromosomes in these cells. Another effect of C-metaphases is the generation of variable chromosome numbers, i.e., the production of disploid cells. Thus, a wide deviation from the normal chromosome number will induce a chromosome imbalance, with loss of viability, partial or complete inactivation of the chromosome spindle, and increased chromosome contraction. C-metaphase should be classified as a mechanism causing serious damage and should be included in the parameters used for the screening of genotoxic and mutagenic substances (Fiskesjö, 1985; Leme and Marin-Morales, 2009).

Another alteration observed during the sequence of mitotic division was the presence of metaphases with chromosome adherence. The herbicide possibly impairs chromosome migration to the poles due to its aneugenic action, leading to an arrest in cell division during metaphase which, in turn, provokes chromosome adherence. Chromosome adherences are a cytogenetic phenomenon that has been widely described in plants. Although first described by Köernicke at the beginning of the last century, the term stickiness was first introduced by Beadle in 1932 to characterize the sticky aspect of corn chromosomes observed in cells carrying a recessive mutation (Fiskesjö, 1993).

All chromosome alterations observed in the present bioassays, such as multipolar anaphases, chromosome bridges, breaks, losses and adherence, micronuclei and C-metaphases, in addition to anaphase and telophase laggards and prophases with loss of genetic material, suggest that Atrazine has a high genotoxicity, with higher concentrations being potentially more genotoxic than lower ones. Since all Atrazine concentrations tested demonstrated genotoxic effects compared to the control test, one may infer that residual doses of this herbicide represent a problem to organisms exposed to it. Thus, Atrazine herbicide can present a potentiality to cause serious damage to human health, due to genotoxicity verified in other eukaryotic organism and, therefore, can present a carcinogenic potentiality for the concentrations tested.

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